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TITLE: METHODS FOR IDENTIFYING GLUCOSE UPTAKE MODULATORS

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METHODS FOR IDENTIFYING GLUCOSE UPTAKE MODULATORS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Patent Application No. 60/391,989, filed June 25, 2002. This entire content of the prior application is incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to methods for identifying agents modulating store-operated Ca^{2+} entry and thereby modulating glucose uptake in mammalian cells. Such compounds may be useful for the treatment of diseases connected with reduced glucose uptake, such as type 2 diabetes.

BACKGROUND ART

Skeletal muscle is quantitatively the most important site of insulin-mediated glucose uptake and this uptake is impaired in patients with type 2 diabetes. Understanding of how insulin increases glucose uptake in skeletal muscle has increased during recent years, but many unresolved questions still remain (Ryder et al. (2001) Acta Physiol Scand 171:249-257). Whether insulin action involves increases in intracellular Ca^{2+} has been debated (Klip and Ramlal (1987) J Biol. Chem. 262:9141-9146; Youn et al. (1994) Am J Physiol 267:R888-R894). Most studies have not been able to detect insulin-mediated increases in free intracellular Ca^{2+} concentration (Klip and Ramlal (1987) J Biol. Chem. 262:9141-9146; Cheung et al. (1987) Am J Physiol 252:C163-C172; Kelly et al. (1989) J Biol. Chem. 264:12754-12757). Furthermore, incubation of isolated rodent skeletal muscles in the presence of nominal $[Ca^{2+}]$ in the incubation medium does not consistently alter insulin-mediated glucose transport (Clausen (1980) Cell Calcium 1:311-325). Indeed a large generalized increase of intracellular Ca^{2+} concentration (e.g. by using Ca^{2+} ionophores) results, if anything, in decreased insulin-mediated glucose uptake (Draznin et al. (1987) J. Biol. Chem. 262:14385-14388; Lee et al. (1995) Am. J. Physiol. 268:R997-R1002).

Recent studies suggest that Ca^{2+} and the Ca^{2+}-binding protein calmodulin are involved in insulin-stimulated glucose transport in skeletal muscle (Shashkin et al. (1995) J Biol Chem 270:25613-25618; Brozinick et al. (1999) Biochem J 339:533-540; Bruton et al. (1999) Proc Natl Acad Sci U S A 96:3281-3286; Bruton et al. (2001) Acta Physiol Scand 171:259-265) as well as in adipocytes (Whitehead et al. (2001) J Biol Chem 276:27816-27824). In skeletal muscle the most direct evidence to support this view lies in the observation that insulin increases the near-membrane free $[Ca^{2+}]$ ($[Ca^{2+}]_{mem}$) and that this increase is attenuated by interventions that inhibit insulin-mediated glucose transport (Bruton et al. (1999) Proc Natl Acad Sci U S A 96:3281-3286). The insulin-induced increase in $[Ca^{2+}]_{mem}$ was inhibited by L-type Ca^{2+} channel blockers. However, the involvement of L-type Ca^{2+} channels remains unclear since these channels are normally activated by depolarization and insulin application causes slight hyperpolarization (Bruton et al., *supra*).

Nucleic acids coding for calcium channel polypeptides are disclosed e.g. in WO 00/40614. Calcium channels are membrane-spanning, multi-subunit proteins that facilitate the controlled transport ("flux") of Ca^{2+} ions into and out of cells. In general, "excitable" cells, such as neurons and skeletal muscle cells, possess voltage-dependent calcium channels. In "non-excitable" cells, calcium influx is thought to occur predominantly in response to stimuli which cause the release of calcium from intracellular stores. This process, termed "store operated" calcium influx, is not well understood.

Recent studies suggest the existence of "store-operated calcium" (SOC), "calcium-release activated calcium" (CRAC) or "store-mediated" Ca^{2+} entry (SMCE) channels in skeletal muscle (Putney et al. (2001) J Cell Sci 114:2223-2229; Kurebayashi and Ogawa (2001) J Physiol 533:185-199). However, the physiological significance of this process is unclear in skeletal muscle as well as in most other excitable cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1B are graphs demonstrating that pharmacological inhibition of "store-mediated" Ca^{2+} entry (SMCE) decreases insulin-stimulated 2-deoxyglucose (2-DG) uptake. 2-DG uptake during insulin (10 milliunits ml^{-1}) exposure in EDL (left) and

soleus (right) muscles is plotted *vs.* the concentration of diphenylboric acid 2-aminoethyl ester (also called 2-aminoethoxydiphenyl borate) (2-APB) (*A*) or cis-*N*-(2-phenylcyclopentyl)azacyclotridec-1-en-2-amine (also called MDL-12,330A) (MDL) (*B*), inhibitors of SMCE. Unfilled circles denote the rate of insulin-stimulated uptake in the absence of SMCE inhibitors. Values are presented as mean ± SEM and the number of muscles tested is indicated for each point. Dashed lines indicate the mean basal rate of 2-DG uptake (i.e. without insulin and SMCE inhibitors).

Figs. 2A-2B are graphs demonstrating that the application of 2-APB decreases the rate of Mn^{2+} quenching of fura-2 fluorescence intensity and the decrease is partially reversed by insulin. 2*A*: Original record of fura-2 fluorescence from a single muscle fiber. Straight lines represent linear fits that were used to measure the rate of decline during periods with a stable decrease rate. 2*B*: Mean data (± SEM; n = 6) of the rate of quenching during exposure to 2-APB and 2-APB + insulin. The rate of quenching before application of 2-APB was set to 100% in each fiber.

Figs. 3A-3b are graphs demonstrating that the rate of Mn^{2+} quenching of fura-2 fluorescence intensity is decreased by application of 2-APB in the presence of insulin and increased after the subsequent washout of 2-APB. 3*A*: Original record from a single muscle fiber; straight lines represent linear fits. 3*B*: Mean data (± SEM) of the relative rate of Mn^{2+} quenching in the type of experiment shown in *A* performed in 5.5 mmol l^{-1} (filled circles; n = 6) and 0 mmol l^{-1} (unfilled circles; n = 5) glucose in the bath solution. The rate of quenching before application of insulin and 2-APB was set to 100% in each experiment (dashed line). 3*C*: Application of insulin had no effect on global free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (measured with indo-1), whereas the subsequent application of 2-APB caused a marked decrease. Record is representative of four experiments.

Fig. 4 is a graph depicting increased 2-DG uptake after 2-APB removal in the continued presence of insulin ($P<0.01$). Mean data (± SEM) from 8 muscles in each group. Dashed line indicates 2-DG uptake under basal conditions (i.e. with out insulin).

Figs. 5A-5B are graphs demonstrating that 2-APB does not inhibit contraction-induced 2-DG uptake. 5A. Representative force record from an EDL muscle exposed to repeated tetanic stimulation (50 Hz, 100 ms duration, 2 contractions/s). 5B. Mean data (±

SEM; n = 5) of 2-DG uptake after the period of repeated contractions in the absence and presence of 2-APB and in rested control muscles.

DISCLOSURE OF THE INVENTION

It has surprisingly been found that "store-mediated" Ca^{2+} entry (SMCE) modulates insulin-stimulated glucose uptake in skeletal muscle. Pharmacological inhibition of SMCE by 2-APB or MDL resulted in a dose-dependent decrease of insulin-mediated 2-deoxyglucose uptake in isolated mouse fast-twitch and slow-twitch skeletal muscles. Neither of these compounds affected basal 2-deoxyglucose uptake. The rate of Mn^{2+} quenching of fura-2 fluorescence intensity was used as an indirect measure of SMCE. Application of 2-APB reduced the rate of Mn^{2+} quenching by about 70% and this decrease was partially reversed by insulin. The effect of 2-APB on Mn^{2+} quenching was larger in the presence than in the absence of extracellular glucose. Removal of 2-APB in the continued presence of insulin increased the rate of Mn^{2+} quenching by about 20% as compared to insulin application on its own. This increase in Mn^{2+} quenching was associated with ~ a 30% increase in insulin-mediated 2-deoxyglucose uptake. 2-APB exposure had no effect on contraction-mediated 2-deoxyglucose uptake. These results support a physiological role of SMCE and Ca^{2+} alterations in insulin action in skeletal muscle, where an increase in Ca^{2+} entry results in an increased insulin-mediated glucose uptake. Thus, SMCE could be a suitable target for future pharmacological treatment of type 2 diabetes.

Consequently, in a first aspect this invention provides a method for identifying an agent that modulates glucose uptake in a mammalian cell, the method comprising: contacting a mammalian cell with a candidate agent; determining whether the candidate agent modulates store-mediated Ca^{2+} entry (SMCE) into the cell; and determining whether the candidate agent modulates glucose uptake in the cell. The method can include, for example, determining whether the candidate agent increases SMCE into the cell. The method can also include determining whether the candidate agent increases glucose uptake in the mammalian cell.

Preferably, the agent increases the open probability of SMCE channels, and thereby the entry of Ca^{2+} and glucose uptake in the cell. Such facilitators of SMCE may

be useful for the treatment of medical conditions involving reduced glucose uptake, such as type 2 diabetes. Candidate agents encompass numerous chemical classes, although typically they are organic compounds. Candidate agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. A candidate agent can contain, for example, a peptide, peptidomimetic, amino acid, amino acid analog, polynucleotide, polynucleotide analog, nucleotide, nucleotide analog, or other small molecule.

In another embodiment, the invention features a method for identifying an agent that modulates glucose uptake in a mammalian cell, the method comprising: contacting a candidate agent with a SMCE-regulating factor; determining whether the candidate agent modulates a function of the SMCE-regulating factor; and determining whether the candidate agent modulates glucose uptake in a mammalian cell. The method can include determining whether the candidate agent stimulates the function of the SMCE-regulating factor. The method can also include determining whether the candidate agent increases glucose uptake in the mammalian cell. The method can also include determining whether the agent binds to the SMCE-regulating factor. The SMCE-regulating factor could in particular be a polypeptide. Examples of SMCE-regulating polypeptides, referred to as "SOC/CRAC calcium channel polypeptides", are disclosed in WO 00/40614. Specifically, the amino acid sequences of human SOC/CRAC calcium channel polypeptides are disclosed as SEQ ID NO: 28, 30 and 32 in WO 00/40614. In the present context, the "functions" include e.g. altering Ca^{2+} entry and glucose uptake in the cell.

In another embodiment, the invention features a method for identifying an agent that modulates glucose uptake in a mammalian cell, the method comprising: contacting a mammalian cell with a candidate, wherein the candidate agent modulates SMCE into the cell; measuring glucose uptake in the cell in the presence of the candidate agent; and determining whether the candidate agent modulates glucose uptake in the cell, wherein altered glucose uptake in the cell in the presence of the candidate agent compared to the absence of the candidate agent indicates that the candidate agent modulates glucose uptake. In one example, the candidate agent increases SMCE into the cell and thereby increases glucose uptake in the cell

Various assays can be used to screen for compounds that modulate the function, as agonists or antagonists, of calcium channels. Several examples of such screening assays have been described; see e.g. U.S. Patent No. 5,643,750.

Fura 2 assay

Human calcium channel expressing animal cells can be employed in the presence of calcium-sensitive, fluorescent dyes (for example fura-2, fluo-3 or indo-1) for measurements of the intracellular calcium concentration after opening and blocking of the calcium channels (See e.g. Rosario et al. (1989) Neurosci. 29: 735-747). The change in the intracellular calcium concentration can in this case be measured by fluorimetry (spectrophotometry). For example, cultured cells can be loaded with fura-2. $MnCl_2$ is then added while measuring the rate of quenching (decline) of fura-2 fluorescence. A drug candidate is then added and any change in the rate of Mn^{2+} quenching is determined (cf. Example 5).

Assay based on genetically-encoded fluorescent Ca^{2+} indicators

In this assay Ca^{2+} is measured with a cameleon, i.e. a fluorescent Ca^{2+} indicator protein. A cameleon consists of a cyan fluorescent protein, calmodulin (CaM), a glycylglycine linker, the CaM-binding domain of myosin light chain kinase, and a yellow fluorescent protein. Ca^{2+} binding to CaM results in a change of the conformation of this molecule and hence altered fluorescence.

Receptor binding assay

Cultured cells transformed with human calcium channels can be cultivated and employed for the preparation of membranes. These membrane preparations can be employed in binding studies with various classes of radioactively labeled substances for screening novel ligands (competitive assay). Examples of known calcium channel binding substances are: phenylalkylamines; benzothiazepines; dihydropyridines; bisphenylbutylpiperidines; and omega conotoxins.

Calcium-45 flux assay

Calcium entry through membranes of cultured cells that have been transformed with human calcium channels can be altered and measured using radioactively labeled calcium (^{45}Ca) (See e.g. Messing et al. (1985) J. Pharmacology and Exp. Therapeutics

235: 407-411) and thereby employed for the functional testing/screening of calcium channel antagonists or agonists.

Electrophysiology

The calcium currents generated SMCE can be measured electrophysiologically (See e.g. Carbone et al. (1990) Pflugers Arch. 416: 170-179). The effect of potential calcium channel antagonists or agonists can be physically measured and pharmacologically characterized directly in human calcium channels using the recombinant animal cell lines.

Indirect methods of measurement

Many cellular processes are controlled by the intracellular calcium ion concentration (for example receptor-mediated signal transmission, various enzyme reactions, such as, for example, phosphorylation, dephosphorylations, neurotransmitter release, Ca-dependent gene regulation etc). Some of these biochemical reactions can be measured using a specific assay. It is thus possible in a recombinant calcium channel-expressing cell system to detect indirectly (physiologically) the effect of calcium channel modulators on calcium-dependent cellular processes (See e.g. Zernig et al. (1986) Eur. J. Pharmacol. 128: 221-229).

It is additionally possible by modifications introduced by targeted mutagenesis, such as, for example, point mutations, insertions, deletions, replacement of DNA segments of various calcium channel subtypes, to detect direct effects on physiological processes (See e.g. Yool and Schwarz (1991) Nature 349: 700-704).

In another aspect, the invention provides a method for modulating glucose uptake in a mammalian cell, the method comprising contacting a mammalian cell with an amount of an agent effective to modulate SMCE and thereby modulate glucose uptake in the cell. In one example, the agent increases SMCE and thereby increases glucose uptake in the cell. The cell can be, for example, a skeletal muscle cell. The method can further include a step of contacting the cell with insulin.

The invention also provides a method for the treatment of type 2 diabetes, the method comprising administering to an individual in need thereof an amount of an agent effective to facilitate SMCE and thereby modulate cellular glucose uptake. The agent can be administered either on its own, or together (simultaneously or subsequently) with the

administration of insulin. The method can further include a step of diagnosing the individual as having type 2 diabetes.

In an additional aspect, the invention provides a method for the treatment of type 2 diabetes, the method comprising: administering to an individual in need of such treatment an amount of an agent effective to sensitize SMCE and thereby modulate cellular glucose uptake; and administering to the individual insulin, subsequently or simultaneously to the administration of the agent. The method can further include a step of diagnosing the individual as having type 2 diabetes.

The term "treatment" means any treatment of a diseases in a mammal, including: (i) preventing the disease, i.e. causing the clinical symptoms of the disease not to develop; (ii) inhibiting the disease, i.e. arresting the development of clinical symptoms; and/or (iii) relieving the disease, i.e. causing the regression of clinical symptoms. The term "effective amount" means a dosage sufficient to provide treatment for the disease state being treated. This will vary depending on the patient, the disease and the treatment being effected.

Throughout this description the terms "standard protocols" and "standard procedures," when used in the context of molecular biology techniques, are to be understood as protocols and procedures found in an ordinary laboratory manual such as: Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994, or Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Suitable methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Below, the invention is described in the appended examples, which are intended to illustrate the invention, without limiting the scope of protection.

RESEARCH DESIGN AND METHODS

Adult male mice (NMRI strain) were housed at room temperature and fed *ad libitum*. Animals were killed by rapid neck disarticulation and muscles removed. Extensor digitorum longus (EDL; mainly fast-twitch fibers)) and soleus (mainly slow-twitch fibers) muscles were used for measurements of 2-deoxyglucose (2-DG) uptake. Flexor digitorum brevis (FDB, mainly fast-twitch fibers) muscles were used in single fiber experiments. All procedures were approved by the local ethics committee.

2-DG uptake was measured essentially as described elsewhere (Shashkin et al. (1995) J Biol Chem 270:25613-25618; Le Marchand-Brustel et al. (1979) J Clin Invest 64:1505-1515). Briefly, muscles were incubated at 35°C in Krebs bicarbonate buffer containing 2 mmol l^{-1} pyruvate (no glucose) +/- an antagonist of SMCE, either 2-APB, a relatively specific inhibitor of SMCE that appears to act directly on SMCE channels (Braun et al. (2001) J Biol Chem 276:1063-1070; Broad et al. (2001) J Biol Chem 276:15945-15952), or MDL, another inhibitor of SMCE with different modes of action as compared to 2-APB (van Rossum et al. (2000) J Biol Chem 275:28562-28568). In other experiments muscles were incubated in 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (TBQ), an inhibitor of SR Ca^{2+} uptake (Westerblad et al. (1994) J Physiol 474:291-301). In some experiments insulin was added (10 milliunits ml^{-1}) after the initial 30 min incubation period. After an additional 30 min, 2-DG (1 mmol l^{-1}; 1 mCi $mmol^{-1}$) and inulin (0.2 μCi per ml medium) were added. Muscles were blotted and frozen in liquid nitrogen 20 min after addition of 2-DG. Muscles were then weighed, digested in NaOH at 70°C, cooled and centrifuged, and aliquots of the supernatants were added to scintillation cocktail and counted for ^{3}H (2-DG) and ^{14}C (inulin).

In some experiments on EDL muscles, 2-DG glucose uptake was measured after 2-APB removal in the continued presence of insulin. After the initial 30 min incubation period, insulin (10 milliunits ml^{-1}) was added and remained in the solution for the rest of the experiment. After an additional 30 min, 2-APB (100 μM) was added. Thirty min later muscles were transferred to medium that did not contain 2-APB and after an additional

10 min, muscles were exposed to 2-DG (as above) for 20 min before being blotted and frozen. Control muscles were treated in the same way except that 2-APB was never added.

The effect of 2-APB on contraction-mediated 2-DG uptake was studied in EDL muscles. The muscles were first stimulated by two tetanic contractions (50 Hz, 100 ms duration) per sec for 10 min. This resulted in a decrease in the glycogen store to less than 20% of the rested value (data not shown). Muscles were allowed to rest for 20 min after the stimulation period and were then exposed to 2-DG for 20 min before being blotted and frozen (as described above). Some muscles were exposed to 2-APB (100 μM) throughout the 40 min recovery period after stimulation.

Experiments on single FDB muscle fibers were performed at 24 °C. The isolated fiber was mounted in a stimulation chamber and continuously superfused by standard Tyrode solution (Lännergren et al. (1987) J Physiol 390:285-293). The free myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured with the fluorescent Ca^{2+} indicator indo-1 (Andrade et al. (1998) J Physiol 509:565-575). An indirect measure of SMCE was obtained by measuring the rate of Mn^{2+} entry and quenching of the fluorescence intensity of fura-2 (Hopf et al. (1996) J Biol Chem 271:22358-22367). $MnCl_2$ (100 μmol l^{-1}) was added to the bath solution and the fura-2 injected muscle fiber was excited at 360 ± 5 nm (the isosbestic wavelength of fura-2 under our experimental conditions) while measuring the fluorescence light emitted at 495 ± 5 nm. The rate of Mn^{2+} quenching was measured during periods where the rate of decrease of the fluorescent light was stable, i.e. ignoring transient changes induced by changes of the bath solution. Measurements were not performed until 2 min after application of insulin, thus allowing time for insulin to act.

Data are presented as mean ± SEM. Statistical analyses were performed as paired or unpaired *t* tests or one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison test. *P* values < 0.05 were considered statistically significant.

EXAMPLES

EXAMPLE 1: Pharmacological inhibition of SMCE decreases insulin-mediated 2-DG uptake

Application of 2-APB decreased insulin-mediated 2-DG uptake in a dose-dependent manner both in EDL and soleus muscles, although the slow-twitch soleus muscles appeared somewhat more sensitive (Fig. 1A). To ensure that this inhibition was not caused by a generalized, unspecific inhibitory effect of the drug, contractile function was tested during exposure to 2-APB. After 30 min exposure to 100 μmol l^{-1} 2-APB, tetanic force was increased by 11 ± 3% (n = 5; $P < 0.05$) and the relaxation speed was slightly decreased; thus, cells were able to generate action potentials in response to electrical stimulation and their contractile machinery was intact. The increase in tetanic force combined with slowed relaxation mimics the situation when the ATP-driven SR Ca^{2+} uptake is inhibited (Westerblad et al. (1994) J Physiol 474:291-301). To ensure that the effect of 2-APB on 2-DG glucose uptake was not due to inhibition of SR Ca^{2+} uptake, we performed control experiments with TBQ. Application of TBQ (500 nmol l^{-1}) resulted in an increase of tetanic force by 4 ± 2% (n = 4; change in force not significantly different from that with 2-APB) and a slight slowing of relaxation. TBQ had no effect on insulin-mediated 2-DG uptake. In soleus muscles insulin-mediated uptake was 163 ± 8 (n = 6), 150 ± 7 (n = 6) and 179 ± 8 (n = 4) μmol l^{-1} min^{-1} without and with 100 nmol l^{-1} and 500 nmol l^{-1} TBQ, respectively. Corresponding values in EDL muscles were 89 ± 5 (n = 6), 93 ± 5 (n = 6) and 103 ± 4 (n = 4) μmol l^{-1} min^{-1}, respectively.

Application of MDL, another SMCE inhibitor, also decreased insulin-mediated 2-DG uptake in a dose-dependent manner both in EDL and soleus muscles (Fig. 1B). As with 2-APB, soleus muscles appeared more sensitive to MDL than EDL muscles. However in contrast to 2-APB, application of 100 μmol l^{-1} MDL resulted in a gradual reduction of tetanic force and after 30 min of exposure force was reduced by 22 ± 2% (n = 4; $P < 0.01$). This reduction was not reversed on washout of the drug.

Mean data for basal 2-DG uptake (i.e. in the absence of insulin) are presented in Table I. Contrary to the insulin-mediated 2-DG uptake, the basal uptake was not significantly affected by application of 2-APB or MDL. TBQ also had no significant

effect on the basal DG uptake. Thus, inhibition of SMCE markedly reduced insulin-mediated 2-DG uptake, whereas the uptake under basal conditions was not affected.

TABLE I: Basal rate of 2-DG uptake

	EDL	Soleus
Control	32 ± 2 (21)	48 ± 3 (20)
2-APB (100 or 1000 μmol l^{-1})	40 ± 4 (7)	55 ± 3 (6)
MDL (300 μmol l^{-1})	42 ± 3 (4)	42 ± 4 (4)
TBQ (500 nmol l^{-1})	37 ± 6 (4)	63 ± 5 (4)

Data (μmol l^{-1} min^{-1}) are means ± SEM (n). The concentration of 2-APB was 0.1 mmol l^{-1} for EDL and 1.0 mmol l^{-1} for soleus.

EXAMPLE 2: Pharmacological inhibition of SMCE decreases the rate of Mn^{2+} quenching

The rate of Mn^{2+} quenching of fura-2 fluorescence was used to assess changes in SMCE activity associated with application of insulin and 2-APB. After addition of Mn^{2+} to the standard Tyrode solution (containing 5.5 mmol l^{-1} glucose), the rate of quenching (expressed as per cent of the initial fluorescence intensity) was 0.72 ± 0.05 % min^{-1} (n = 12). Figure 2A shows original records of the fura-2 fluorescence during a quenching experiment where a single muscle fiber was exposed to 2-APB (100 μmol l^{-1}) and subsequently to insulin (10 mU ml^{-1}) in the continued presence of 2-APB. It can be seen that the rate of quenching was markedly reduced when 2-APB was added and partially recovered after application of insulin. Similar results were obtained in five more experiments and mean data are shown in Fig. 2B. Thus, application of 2-APB decreased the rate of quenching by about 70% and insulin restored about half of this decrease. The marked decrease of the rate of quenching with 2-APB application would reflect a proportional reduction in the rate of Ca^{2+} influx into the cells, which might affect $[Ca^{2+}]_i$. Therefore, we measured changes in global $[Ca^{2+}]_i$ (using indo-1) in association with 2-APB exposure. Application of 2-APB caused a clear decrease of $[Ca^{2+}]_i$ from 70 ± 10 to 51 ± 9 nmol l^{-1} (n = 5; $P < 0.01$).

Another series of quenching experiments was performed in which insulin was first applied and then 2-APB was added. Figure 3A shows original records from one such experiment where insulin exposure resulted in a slight increase in the rate of quenching. An increased rate of quenching was observed in one more fiber, whereas the other four fibers tested showed a decrease or no change at all. Thus, mean data showed no significant insulin-induced change of the rate of quenching (Fig. 3B, filled circles). The subsequent exposure to 2-APB in the presence of insulin resulted in a marked reduction of the rate of quenching. Interestingly, removal of 2-APB in the continued presence of insulin resulted in a rate of quenching that was significantly (about 20%; $P < 0.05$) higher than the original. $[Ca^{2+}]_i$ was measured in a separate series of experiments (Fig. 3C). Mean data (n = 4) showed no change of global $[Ca^{2+}]_i$ when insulin was applied (75 ± 13 *vs.* 74 ± 13 nmol l^{-1}), whereas the subsequent application of 2-APB resulted in a rapid and significant ($P < 0.05$) decrease of $[Ca^{2+}]_i$ (56 ± 9 nmol l^{-1}) that was reversed on washout of insulin and 2-APB (76 ± 12 nmol l^{-1}).

Since all quenching experiments described thus far experiments were performed with glucose in the incubation buffer, a series of Mn^{2+} quenching experiments was performed in Tyrode solution with 0 mmol l^{-1} glucose. The basal rate of quenching in zero glucose (0.64 ± 0.06% min^{-1}; n = 5) was not significantly different from that in 5.5 mmol l^{-1} glucose (see above). On the other hand, addition of insulin in zero glucose resulted in a small (about 10%) increase in the rate of quenching in all fibers tested (Fig. 3B; unfilled circles). The response to addition and removal of 2-APB was qualitatively similar in zero and 5.5 mmol l^{-1} glucose, although the rate of quenching was generally higher in the former. The reason for this difference was not further investigated.

The contractile response to tetanic stimulation (70 Hz, 350 ms duration) was tested before and after exposures to 2-APB in most of the above Mn^{2+} quenching experiments and neither tetanic $[Ca^{2+}]_i$ nor force showed any noticeable changes (data not shown).

Removal of 2-APB in the continued presence of insulin results in increased 2-DG uptake. Fig. 3B shows that the rate of Mn^{2+} quenching is increased after removal of 2-APB. Having this in mind, we measured the rate of 2-DG uptake in EDL muscles under similar experimental conditions. The results showed a marked (~30%) increase in 2-DG

uptake in muscles previously exposed to 2-APB and insulin as compared to insulin alone (Fig. 4).

EXAMPLE 3: 2-APB exposure does not affect contraction-mediated 2-DG uptake

In addition to insulin, repeated contractions induce a marked increase in glucose uptake in skeletal muscle. To test if 2-APB also affected contraction-mediated 2-DG uptake, EDL muscles were exposed to intense, repeated tetanic stimulation for 10 min and then allowed to recover in the presence or absence of 2-APB. The stimulation protocol decreased force production to ~10% of the original (Fig. 5A) and increased 2-DG uptake about 3-fold both in the presence and absence of 2-APB (Fig. 5B).

Thus, SMCE appears not to be involved in this alternative way to activate glucose transport. It should be noted that repeated contractions as such result in a marked increase in $[Ca^{2+}]_i$. This increase may be sufficient to support glucose transport and hence activation of SMCE would not be required.

EXAMPLE 4: Identification of agents modulating glucose uptake

Agents modulating the activity of SMCE and the rate of glucose uptake in human cells can be identified by methods known in the art, e.g. by any of the following methods:

Quenching assay

This assay will indirectly measure the rate of Ca^{2+} influx by measuring the rate of Mn^{2+}-quenching of fura-2 fluorescence (cf. Example 2). Briefly, cultured cells are loaded with fura-2AM in the standard fashion (e.g., Basavappa et al. (1999) Biochem Biophys Res Comm 254:699-702). After loading they are allowed to rest for about 1 hour to make sure that all fura-AM has been converted to the active form. $MnCl_2$ (100-500 μM) is then added to the bath solution while measuring the rate of quenching (decline) of fura-2 fluorescence (excitation and emission wavelengths as in Example 2) for about 10 min. A drug candidate is then added to the bath +/- insulin, and possible changes in the rate of Mn^{2+} quenching are measured. Increased quenching suggests increased Ca^{2+} influx, which may reflect increased glucose uptake.

Assay based on genetically-encoded fluorescent Ca^{2+} indicators

In this assay Ca^{2+} is measured with a cameleon, i.e. a fluorescent Ca^{2+} indicator protein. A cameleon consists of a cyan fluorescent protein, calmodulin (CaM), a glycylglycine linker, the CaM-binding domain of myosin light chain kinase, and a yellow fluorescent protein. Ca^{2+} binding to CaM results in a change of the conformation of this molecule and hence altered fluorescence. The cameleons can be targeted to specific intracellular sites by fusing them to specific host proteins (e.g. Miyawaki et al. (1997) Nature 388: 882-887). In this assay the cameleon will initially be fused to some surface membrane protein. Ultimately, it will be fused to the target that is the likely candidate to be activated by insulin.

Cultured cells are infected by adenoviral vectors of the cameleon YC2.1 as described in Miyawaki et al. (1999) Proc. Natl. Acad. Sci. U.S.A. 96: 2135-2140. The vectors are modified so that they will fuse with a suitable surface membrane host protein (see above). Ca^{2+} concentration is measured by the cells being excited at 432 nm while measuring the light emitted at 535 nm and 480 nm. Increases in the 535 nm / 480 nm ratio reflects increased Ca^{2+} concentration. To obtain a qualitative measure of changes in Ca^{2+} concentration (i.e. it goes up or down), it would be possible to only measure at one of the above emission wavelengths. Ca^{2+} concentration will be measured under control conditions and after addition of a drug candidate +/- insulin.

OTHER EMBODIMENTS

It is to be understood that, while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications of the invention are within the scope of the claims set forth below.

What is claimed is: